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(54) Variable fragments of immunoglobulins - use for therapeutic or veterinary purposes

(57) The present invention relates to fragments, especially variable fragments of immunoglobulins which are by nature devoid of light chains these fragments being nevertheless capable of exhibiting a recognition and binding activity toward specific antigens.

The present invention further relates to the use of such immunoglobulin fragments formed of at least one heavy chain variable fragment or derived therefrom, for therapeutic or veterinary purposes and especially for passive immunotherapy or serotherapy.

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Description

The present invention relates to fragments, especially variable fragments of immunoglobulins which are by nature devoid of light chains these fragments being nevertheless capable of exhibiting a recognition and binding activity toward specific antigens. These fragments of immunoglobulins can for example be obtained by the expression in host cells for example in prokaryotic cells or eukaryotic cells of nucleotide sequences obtained from animals naturally expressing so-called "two-chain immunoglobulins", for instance from animals of the camelid family.

The present invention further relates to the use of such immunoglobulin fragments formed of at least one heavy chain variable fragment or derived therefrom, for therapeutic or veterinary purposes and especially for passive immunotherapy or serotherapy.

Functional immunoglobulins devoid of light polypeptide chains termed « two-chain immunoglobulin » or « heavy-chain immunoglobulin » have been obtained from animals of the family of camelids and have been described in an international patent application published under number WO 94/04678, together with two publications, especially Hamers-Casterman et al, 1993 and Muyldermans et al, 1994).

The isolation and characterization of these immunoglobulins, together with their cloning and sequencing have been described in the above referrenced documents which are incorporated by reference in the present application.

According to WO 94/04678 it has been established that different molecules can be isolated from animals which naturally produce them, which molecules have functional properties of the well known four-chain immunoglobulins these functions being in some cases related to structural elements which are distinct from those involved in the function of four-chain immunoglobulins due for instance to the absence of light chains.

These immunoglobulins having only two chains, neither correspond to fragments obtained for instance by the degradation in particular the enzymatic degradation of a natural four-chain model immunoglobulin, nor correspond to the expression in host cells, of DNA coding for the constant or the variable regions of a natural four-chain model immunoglobulin or a part of these regions, nor correspond to antibodies produced in lymphopaties for example in mice, rats or human.

The immunoglobulins devoid of light chains are such that the variable domains of their heavy chains have properties differing from those of the four-chain immunoglobulin variable heavy chain (V_H) For clarity reasons, this variable domain according to the invention will be called V_{HH} in this text to distinguish it from the classical V_H of four-chain immunoglobulins.The variable domain of a heavy-chain immunoglobulin according to the invention has no normal interaction sites with the V_L or with the C_H1 domain which do not exist in the heavy-chain immunoglobulins. It is hence a novel fragment in many of its properties such as solubility and conformation of main chains. Indeed the V_{HH} of the invention can adopt a three-dimensional organization which distinguishes from the three-dimensional organization of known four-chain immunoglobulins according to the description which is given by Chothier C. and Lesk A.M, (1987- J.Mol. Biol. 197, 901-917).

According to the results presented in patent application WO 94/04678, the antigen binding sites of the isolated immunoglobulins, naturally devoid of light chains are located on the variable region of their heavy chains. In most cases, each heavy chain variable region of these two-chain immunoglobulins can comprise an antigen binding site.

A further characteristic of these two-chain immunoglobulins is that their heavy polypeptide chains contain a variable region (V_{HH}) and a constant region (C_H) according to the definition of Roitt et al but are devoid of the first domain of the constant region is called C_H1.

These immunoglobulins of the type described hereabove can comprise type G immunoglobulins and especially immunoglobulins which are termed immunoglobulins of class 2 (IgG2) or immunoglobulins of class 3 (IgG3), according to the classification established in patent application WO 94/04678 or in the publication of Muyldermans et al (Protein Engineering Vol.7, N°9, pp 1129-1135-1994).

The absence of the light chain and of the first constant domain lead to a modification of the nomenclature of the immunoglobulin fragments obtained by enzymatic digestion, according to Roitt et al.

The terms Fc and pFc on the one hand, Fc' and pFc' on the other hand corresponding respectively to the papain and pepsin digestion fragments are maintained.

The terms Fab, $F(ab)_2$,$F(ab')_2$,Fabc, Fd and fv are no longer applicable in their original sense as these fragments have either a light chain, the variable part of the light chain or the CH_1 domain.

The fragments obtained by papain digestion or by V8 digestion, composed of the V_{HH} domain of the hinge region will be called $FV_{HH}h$ or $F(V_{HH}h)2$ depending upon whether or not they remain linked by the disulphide bonds.

The immunoglobulins referring to the hereabove given definitions can be originating from animals especially from animals of the camelid family. These heavy-chain immunoglobulins which are present in camelids are not associated with a pathological situation which would induce the production of abnormal antibodies with respect to the four-chain immunoblobulins. On the basis of a comparative study of old world camelids (Camelus bactrianus and Camelus dromaderius) and new world camelids (for example Lama Paccos, Lama Glama, and Lama Vicugna) the inventors have shown that the immunoglobulins devoid of light polypeptide chains are found in all species. Nevertheless differences may be apparent in molecular weight of these immunobglobulins depending on the animals. Especially the molecular

weight of a heavy chain contained in these immunoglobulins can be from approximately 43 kd to approximately 47 kd, in particular 45 kd.

Advantageously the heavy-chain immunoglobulins of the invention are secreted in blood of camelids.

The variable fragments of heavy chains of Immunoglobulins devoid of light chains can be prepared starting from immunoglobulins obtainable by purification from serum of camelids according to the process for the purification as described in detail in the examples of WO 94/04678. These fragments can also be generated in host cells by genetic engineering or by chemical synthesis. They can also be obtained from heavy-chain immunoglobulins by digestion with papain or V8 enzymes.

The observation by the inventors that Camelidae produce a substantial proportion of their functional immunoglobulins as a homodimer of heavy chains lacking the C_H1 domain and devoid of light chains(Hamers-Casterman et al, 1993), led to the proposal of having recourse to an immunized camel to generate and select single variable antibody fragments (V_{HH}).

Cloned camel single V_{HH} fragments were displayed on bacteriophages for selection and in bacteria for the large scale production of the soluble proteins, and were shown to possess a superior solubility behaviour and affinity properties compared to the mouse or human V_H equivalents (Muyldermans et al, 1994). Following this strategy, one would obtain small ligand binding molecules (MW around 16,000 D) which are not hindered by the presence of an oligopeptide linker (Borrebaeck *et al.*, 1992) or not inactivated by the disassembly of the VH-VL complex (Glockshuber *et al.*, 1990). The camel V_{HH} fragments have the additional advantage that they are characteristic of the heavy chain antibodies which are matured in vivo in the absence of light chains.

The inventors have obtained evidence that variable fragments of high chains of immunoglobulins devoid of light chains can display an effective therapeutic activity when they are generated against a determined antigen.

To develop this technology of preparing and identifying usefull camel V_{HH} fragments, it is critical (i) that camels can be immunized with a variety of antigens, (ii) that the camel V_{HH} genes can be cloned and expressed on filamenteous phages and in E.coli for easy selection with the immobilized antigen by panning, (iii) that the expressed camel V_{HH}'s are properly folded, and (iv) that they have good solubility properties and possess high affinities and specificities towards their antigen.

Camel V_{HH} genes derived from the heavy chain immunoglobins lacking the light chains were previously cloned and analysed (Muyldermans et al., 1994). A comparison of the amino acid sequences of these camel V_{HH} clones clearly showed that the key features for preserving the characteristic immunoglobulin fold are all present. The specific amino acid replacements observed in the camel V_{HH} clones could correlate with the absence of the VL (variable light chains) and the functionality of the camel single V_{HH} domain (Muyldermans et al., 1994).

The invention thus relates to a variable fragment (V_{HH}) of a heavy chain of an immunoglobulin devoid of light chains, which is encoded by a nucleotide sequence obtainable by the following process:

- treating blood lymphocytes or other appropriate cells of an animal of the Camelid family previously immunized with a determined antigen, in order to give access to their mRNA,
- synthesizing a first strand of cDNA starting from the obtained mRNA,
- contacting the obtained cDNA with at least two different primer oligonucleotides in conditions allowing their hybridization to at least two complementary nucleotide sequences contained in the cDNA, said primers comprising a BACK primer (back p1) having the following nucleotide sequence 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3' and a FOR primer (forp 1) replying to the following nucleotide sequence 5'-CGCCATCAAGGTACCGTTGA-3'
- amplifying the DNA fragment located between the nucleotide sequence hybridized with said primers and,
- recovering amplified DNA corresponding to bands of different size orders including:
  . a band of around 750 basepairs which is the amplified product of the variable heavy chain (V_H), CH1, hinge and part of CH2 region of a four-chain immunoglobin,
  . a band of around 620 basepairs which is the amplified product of the variable heavy-chain (V_{HH}), long hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG2,
  . a band of around 550 basepairs which is the amplified product of the variable heavy-chain (V_{HH}), short hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG3,
- purifying the two shortest bands from agarose gel, for example by Gene Clean,
- recovering the amplified DNA fragments containing nucleotide sequences encoding the V_{HH} fragments,
- digesting the amplified products with restriction enzymes having target sites within the amplified fragments and/or in the nucleotide primers, for example with PstI and BstEII,
- recovering the digested amplified DNA fragments,
- ligating the amplified DNA fragments to a phasmid vector, for example in a pHEN4 vector, in conditions allowing the expression of the amplified fragments when the obtained recombinant vector is used to transform a host cell,

- transforming a determined bacterial host cell for example an E. Coli cell with the obtained recombinant phasmid vector, and growing the cells on selective medium, to form a library,
- infecting the obtained library of recombinant host cells after culture in an appropriate selective medium, with bacteriophages, for instance M13K07 bacteriophages to obtain recombinant phagemid virions,
- incubating the recombinant host cells in conditions allowing secretion of recombinant phagemid virions particles containing the recombinant phasmid, for instance the pHEN4 phasmid packaged within the M13 virion.
- isolating and concentrating the recombinant phagemid virions,
- submitting the phagemid virions to several rounds of panning with the antigen of interest previously immobilized, in conditions allowing the adsorption of the phagemid virions on the immobilized antigen,
- eluting the adsorbed phagemid virions, and growing them on appropriate cells,
- amplifying the phagemid virions by infecting the cells with helper bacteriophage,
- recovering the virions and testing them for their binding activity against the antigen of interest, for example by ELISA,
- recovering the phagemid virions having the appropriate binding activity,
- isolating the nucleotide sequence contained in the phasmid vector and capable of being expressed on the phagemid virions as a V_{HH} aminoacid sequence having the appropriate binding activity.

In a preferred embodiment of the invention, the variable V_{HH} fragments are obtainable by adding to the hereabove described amplification step of the cDNA with BACK and FOR primers (p1), a further amplification step with a BACK primer corresponding to the oligonucleotide sequence which has been described hereabove (back p1) and the FOR primer (for p2) having the following nucleotide sequence: 5'- CG ACT AGT GCG GCC GCG TGA GGA GAC GGT GAC CTG-3'. Not and BstEII sites which can be used for cloning in the pHEN4 vector have been underlined. This FOR primer allows hybridization to the codon position of framework 4 (FR4) region of the V_{HH} nucleotide sequences (amino acid position 113-103).

According to another variant of the process described, this additional amplification step can replace the amplification step which has been described with BACK primer and a FOR primer having respectively the following nucleotide sequences:

5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3'

5'-CGCCATCAAGGTACCGTTGA-3'

The restriction sites have been underlined.

In another embodiment of the invention the amplification step of the synthetized cDNA is performed with oligonucleotide primers including hereabove described BACK primer and FOR primer having the following sequences:

FOR primer 3: 5'- TGT CTT GGG TTC TGA GGA GAC GGT -3'

FOR primer 4: 5'- TTC ATT CGT TCC TGA GGA GAC GGT -3'

According to this latter embodiment, the V_{HH} fragments of the invention are immediately and specifically amplified by a single amplification (for instance PCR reaction) step when the mixture of FOR primers is used.

These latter primers hybridize with the hinge/framework 4 and short hinge/framework 4 respectively. Each of these FOR primers allows the amplification of one IgG class according to the classification given in patent application WO 94/04678.

The variable V_{HH} fragments corresponding to this definition can also be obtained from other sources of animal cells, providing that these animals are capable of naturally producing immunoglobulins devoid of light chains according to those described in the previous patent application WO 94/04678.

These variable fragments (V_{HH}) can also be obtained by chemical synthesis or by genetic engineering starting from DNA sequences which can be obtained by the above described process.

The variable fragment of a heavy chain of an immunoglobulin devoid of light chains according to the preceding definitions is specifically directed against an antigen against which the animal has been previously immunized, either by natural contact with this antigen or by administration of this antigen in order to generate an immune response directed against it.

The process which is proposed hereabove to prepare a nucleotide sequence coding for the variable fragments of the invention contains steps of phage display library construction which allow the selection of nucleotide sequences coding for variable fragments of heavy chains having the desired specificity.

According to one preferred embodiment of the invention, the variable fragments of a heavy chain of a immunoglobulin devoid of light chains according to the invention is obtainable from an animal having been previously immunized with a toxin, especially a toxin of a bacteria or a part of this toxin sufficient to enable the production of immunoglobulins directed against this toxin and especially immunoglobulins devoid of light chains.

According to another embodiment of the invention, the variable fragments of a heavy chain of a immunoglobulin devoid of light chains according to the invention is obtainable from an animal having been previously immunized with substances contained in venom of animals.

The antigen used for immunization of the animals is usually under a non toxic form.

The variable fragments according to the invention can be derived from immunoglobulins belonging to different classes especially belonging to IGg2 or IGg3 immunoglobulin classes, according to the classification given in patent application WO/04678.

In a preferred embodiment of the invention, the variable fragment of a heavy-chain of an immunoglobulin devoid of light chains is directed against the tetanus toxin of Clostridium tetani or against a fragment thereof.

The variable fragments of heavy chains of immunoglobulins devoid of light chains can be also generated against toxins or part thereof from pathogenic organisms such as bacteria and especially can be chosen among the toxins or toxoids of the following bacteria: Clostridium, especially Clostridium Botulinum or Clostridium Perfringens, Staphylococcus, Pseudomonas, Pasteurella, Yersinia, Bacillus Anthracis, Neisseria, Vibrio, especially Vibrio cholera, enterotoxic E. Coli, Salmonella, Shigella, Listeria.

Other antigens appropriate for the preparation of the V_{HH} fragments of the invention can be obtained from the following organism: anemonies, coral, jellyfish, spiders, beas, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.

According to another embodiment of the invention, the variable fragment V_{HH} of a heavy chain of an immunoglobulin devoid of light chains is characterized in that it comprises the following aminoacid sequences:

GluValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGlyGlySerLeuArgLeuSer

CysAlaAlaSerGly(**CDR1**)Trp(Phe/Tyr)ArgGlnAlaProGlyLysGlu(Arg/Cys)Glu

(Gly/Leu)ValSer(**CDR2**)ArgPheThrIleSerArgAspAsnAlaLysAsnThrVal

TyrLeuGlnMetAsnSerLeuLysProGluAspThrAlaValTyrTyrCysAlaAla(**CDR3**)

TrpGlyGlnGlyThrGlnValThrValSerSer

wherein CDR1, CDR2 and CDR3 represent variable amino acid sequences providing for the recognition of a determined epitope of the antigen used for the immunization of Camelids, CDR1, CDR2 and CDR3 sequences comprising from 5 to 25 amino acid residues preferably CDR1 contains from 7 to 12 amino acid residues, CDR2 contains from 16 to 21 amino acid residues and CDR3 contains from 7 to 25 amino acid residues.

The camel V_{HH} specific amino acid residues Ser 11, Phe 37, Glu 44, Arg 45, Glu 46, Gly 47 are underlined.

One preferred variable fragment according to the invention is encoded by a nucleotide sequence present in recombinant phasmid pHEN4-αTT2(WK6) deposited at the BCCM/LMBP (Belgium) under accession number LMBP3247.

The pHEN4αTT2 (described on Figure 2) is a phasmid carrying a PelB leader signal, a camel V_{HH} gene of which the protein binds tetanus toxoid, a decapeptide tag (from ImmunoZAP H of Stratacyte) and gene IIIp of M13 in the pUC 119 polylinker between the HindIII and EcoRI sites. This phasmid was transformed in E. coli WK6 cells.

A specific variable fragment according to the invention is for instance characterized in that it comprises the following αTT1 aminoacid sequence:

GluValGlnLeuGlnAlaSerGlyGlyGly***Ser***ValGlnAlaGlyGlySerLeuArgLeu

SerCysAlaAlaSerGlyGlyGlnThrPhe***AspSerTyrAlaMetAla***Trp***Phe***ArgGln

AlaProGlyLysGlu*Cys*GluLeuValSer***SerIle***IleGly***AspAspAsnArgAsnTyr***

AlaAspSerValLysGlyArgPheThrIleSerArgAspAsnAlaLysAsnThrValTyr

LeuGlnMetAspArgLeuAsnProGluAspThrAlaValTyrTyrCysAlaGln***LeuGly***

SerAlaArgSerAlaMetTyrCysAlaGlyGlnGlyThrGlnValThrValSerSer

According to another preferred embodiment of the present invention, the variable fragment comprises the following αTT2 aminacid sequence :

GluValGlnLeuGlnAlaSerGlyGlyGly***Ser***ValGlnAlaGlyGlySerLeuArgLeu

SerCysThrAlaAlaAsnTyrAlaPheAspSerLysThrValGlyTrpPheArgGlnVal

ProGlyLysGlu***Arg***GluGlyValAla***GlyIleSerSerGlyGlySerThrThrAlaTyr***

SerAspSerValLysGlyArgTyrThrValSerLeuGluAsnAlaLysAsnThrValTyr

LeuLeuIleAspAsnLeuGlnProGluAspThrAlaIleTyrTyrCysAlaGly***ValSer***

GlyTrpArgGlyArgGlnTrpLeuLeuLeuAlaGluThrTyrArgPheTrpGlyGlnGly

ThrGlnValThrValSerSer

In a preferred embodiment of the invention, the variable V_{HH} fragment of the invention is altered in order to diminish its immunogenic properties. Such a modification can lead to an alternated immunological reaction against the V_{HH} fragments of the invention when they are administered to a host either human or animal, for passive immunoprotection for example.

The invention further relates to a pharmaceutical composition comprising an immunoglobulin heavy chain variable fragment according to those which have been defined hereabove, in admixture with a physiologically acceptable vehicle.

Such pharmaceutical composition can be used for the treatment by passive immunisation, of infections or acute intoxications by toxins such as those of Clostridium, especially Clostridium Botulinum or Clostridium Perfringens, Staphylococcus, Pseudomonas, Pasteurella, Yersinia, Bacillus Anthracis, Neisseria, Vibrio, especially Vibrio cholera, enterotoxic E. Coli, Salmonella, Shigella, Listeria or anemonies, coral, jellyfish, spiders, beas, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.

The present invention further relates to nucleotide sequences coding for a variable fragment (V_{HH}) of a heavy chain of an immunoglobulin devoid of light chains, obtainable by the process which has been described hereabove.

Specific nucleotide sequences are those corresponding to αTT1 and αTT2 as described on figures 4A and 4B.

According to an embodiment of the invention, a preferred nucleotide sequence is the sequence contained on phasmid pHEN4-αTT2 deposited at the BCCM/LMBP collection in Belgium on January 31, 1995 under no. LMBP3247.

Other characteristics of the invention will appear from the figures and the examples which are described hereafter.

FIGURE 1: 1% agarose gel electrophoresis of the PstI/BstEII digested PCR amplification product of the camel V_{HH} gene (lanes 1 and 2) next to the 123 bp ladder of BRL used as a size marker (lane 4). The PCR product comigrates with the 3rd band of the marker, 369 bp in length.

FIGURE 2: Map of the pHEN4 with the nucleotide sequence of the V_{HH} cloning site shown in the lower part of the figure. The PstI and BstEII sites can be used to clone the camel V_{HH} PCR product shown in Figure 1.

FIGURE 3: 100 individual clones were randomly selected from the original camel V_{HH} library (0), or after the first (1), second (2), third (3) or fourth (4) round of panning. After M13 infection the virions were tested for binding activity against immobilized tetanus toxoid. The number of positive clones are shown as a function of number of pannings.

FIGURE 4: Nucleotide sequence and the corresponding amino acid sequence of the two identified camel V_{HH} anti tetanus toxoid clones pHEN4-αTT1 and pHEN4-αTT2. The framework SerII, Phe37 and Arg or Cys 45 characteristic for the camel V_{HH} heavy chain antibodies (Muyldermans et al, 1994) are double underlined. The three hypervariable or CDR's according to Kabat et al, (1991) are underlined.

FIGURE 5: SDS-polyacrylamide gel electrophoresis of the proteins extracted from the periplasm of WK6 cultures induced with IPTG. Lane 1 & 8, protein size marker (Pharmacia) MW are (from top of to bottom) 94,000; 67,000; 43,000; 30,000; 20,100 and 14,400 D. Lanes 2 and 7 Expressed periplasmic proteins extracted from WK6 cells containing pHEN4-αTT2' and pHENA-αTT1' cloning vector. Lane 3 & 4, Purified V_{HH} domain of pHEN4-αTT2 at 10 and 1 microgram. Lanes 5 & 6, Purified V_{HH} domain of pHEN4-αTT1 at 10 and 1 migrogram. The position of the expressed soluble camel VH protein is indicated with an arrow. It is clearly absent in the second lane.

FIGURE 6: The total periplasmic extract of 1 liter of culture of WK6 cells carrying the pHEN4-αTT2 was concentrated to 5 ml and fractionated by gel filtration on Superdex 75 (Pharmacia) using 150mM NaCL, 10 mM sodiumphosphate pH7.2 as eluent. The pure V_{HH} is eluted at the fractions between the arrows.

FIGURE 7: CD (Circular dichroism) spectrum (Absorbance versus wavelength in nm) of the purified V_{HH} domain αTT2 at 3.9×10^{-6} Min water measured in a cuvette with a pathlength of 0.2 cm. The negative band near 217 and 180 nm and the positive band around 195 nm are characteristic for β structures (Johnson, 1990).

FIGURE 8: Specificity of antigen binding shown by competitive ELISA. The experiments were carried out in triplicate with the bacterial periplasmic extracts of pHEN4-αTT1 and pHEN4-αTT2.

FIGURE 9: Number of mice surviving after I.P injection of 100 ngr tetanus toxin (10 x LD50) or co-injection of tetanus toxin with the purified V_{HH}αTT1, αTT2 or the non-specific cVH21 (Muyldermans et al., 1994) at 4 or 40 microgram.

FIGURE 10: Variability plot of the camelid V_{HH} sequence (CDR3 and framework 4 regions are not included).

The alignment of the V_{HH} amino acid sequences of camel and lama (a total of 45 sequences) was performed according to Kabat et al. The variability at each position was calculated as the number of different amino acids occuring at a given position, divided by the frequency of the most common amino acid at that position. Positions are numbered according to Kabat et al. The positions above the horizontal bar indicate the amino acids which are referred to as (CDR1) and (CDR2) in the consensus sequence.

A variability number equal to 1 indicates a perfectly conserved amino acid at that position. The higher the variability number the more likely it will be that the amino acid at this position will deviate from the consensus sequence.

Examples:

Generation of specific camel V_{HH} fragments

In this application, results are presented, which prove the feasibility of generating specific camel V_{HH} fragments with demonstrated folding and good binding affinity. This was done by generating a library of camel V_{HH} fragments derived from the dromedary IgG2 and IgG3 isotype, display of the V_{HH} library on phage as fusion proteins with the gene III protein of bacteriophage M13 to allow selection of the antigen binders, and finally of expressing and extracting the

soluble and functional V_{HH} fragments from E.coli. As antigen, we choose the tetanus toxoid was chosen because comparisons are possible with published data. In addition, the tetanus toxoid is a highly immunogenic protein that is routinely used as a vaccine in humans to elicit neutralizing antibodies.The two camel V_{HH} fragments that were identified were specific and of high affinity. The affinities of the two camel V_{HH} fragments appear to be comparable with those from the human anti-tetanus toxoid F_{AB}'s recently obtained by Mullinas et al. (1990) and by Persson et al. (1991).

Camel Immunization

The serum of a camel (Camelus dromedarius) was shown to be non-reacting with tetanus toxoid (RIT,Smith Kline Beecham, Rixensart, Belgium). This camel was injected with 100 µgr tetanus toxoid at days 9, 30,52, 90 and with 50 µgr at days 220, 293 and 449. The blood was collected 3 days after each injection.

mRNA purification of camel blood lymphocytes

Peripheral blood lymphocytes were purified with Lymphoprep (Nycomed, Pharma) from the bleeding at day 452. Aliquots of 1.10^6 -5.10^6 cells were pelleted and frozen at - 85°C and subsequently used as an enriched source of B-cell mRNA for anti-tetanus toxoid.

The mRNA was prepared from a total of 10^6 peripheral blood lymphocytes either by the "Micro FastTrack" mRNA isolation kit (Invitrogen) or the "QuickPrep Micro mRNA Purification" kit of Pharmacia, following the recommendations of the manufacturer. With both protocols, up to a few µgr of mRNA was obtained which was used in the subsequent cDNA synthesis step.

cDNA synthesis and PCR amplification of camel V_{HH} gene

The first-strand cDNA was synthesized with the Invitrogen "cDNA-cycle" or the Pharmacia "Ready-To-Go"kit. The first-strand cDNA was used immediately afterwards for the specific amplification of the camel V_{HH} region by PCR. The primers used have following sequences : the BACK primer (5'-GA TGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3'), the internal PstI site is underlined) is designed to hybridize tothe framework 1 region (codons 1 to 10) of the camel V_{HH}, while the FOR primer (5'-CGCCATCAAGGTACCGTTGA-3') hybridizes in the CH2 region. The PCR was carried out with the Taq polymerase from Boehringer Mannheim.

The PCR product was purified according to standard protocols (Sambrook et al., 1989) and digested with the PstI restriction enzyme of which the target site occurred in the BACK primer, and with BstEII which has a naturally occurring site in the framework 4 of the camel V_{HH} regions. The resulting fragments of approximately 360 bp (FIGURE 1) were ligated into the pHEN4 vector cut with the same restriction enzymes. The pHEN4 vector (FIGURE 2) is the pHEN1 phasmid (Hoogenboom et al., 1991) - a pUC119 based vector -where the myc-tag was replaced by the decapeptide tag present in the ImmunoZAP H vector (Stratacyte). Also the polylinker was modified to allow the cloning of the camel V_{HH} gene between a PstI and a BstEII site located after the PelB leader signal and in front of the decapeptide tag and gene III of bacteriophage M13.

Construction of a camel V_{HH} library

The ligated DNA material was precipitated with 10 volumes and resuspended in 10 ml water and electrotransformed in E.coli XL1 Blue MRF' cells (Stratagene). After electroporation according to the recommended protocol (Stratagene) we kept the cells for 1 hour at 37°C in 1 ml SOC medium before plating on LB plates containing 100 mg ampicilline/ml. After an over night incubation at 37°C the transformed cells were grown out into colonies and some 500,000 recombinant clones were obtained. About 20 colonies, randomly selected, were toothpicked and grown in selective medium (LB/Ampicilline) to prepare plasmid DNA and to check their insert by sequencing. For each clone tested, we found a different V_{HH} region with the aminoacid sequence and contents characteristic for a V_{HH} originating from a camel heavy chain immunoglobulin (Muyldermans et al., 1994). This indicates that a vast camel V_{HH} library was generated.

The remaining 500,000 clones were scraped from the plates with a minimal amount of LB containing 50% glycerol and stored at -85°C until further use.

Panning with tetanus toxoid

The library was screened for the presence of anti-tetanus toxoid camel V_{HH}'s by panning. To this end, approximately 10^9 cells (=5 ml suspension of the frozen recombinant clones) were grown to midlogarithmic phase in 200 ml of LB medium supplemented with 1% glucose and 100 µg ampicilline/ml before infection with M13K07 bacteriophages. After adsorption of the bacteriophages on the E.coli cells for 30 min at room temperature, the cells were harvested by

centrifugation and washed in LB medium supplemented with ampicilline and kanamycin (25μg/ml).The cells were incubated overnight at 37°C to secrete the recombinant pHEN phasmid packaged within the M13virion containing a camel V_{HH} fused to some of its M13gene III proteins (Hoogenboom et al., 1991). The phagemid virions were prepared according to the protocol described by Barbas et al. (1991). The phage pellets were resuspended in blocking solution (1% casein in phosphate buffered saline, PBS), filtered through a 0.2 mm filter into a sterile tube and used for panning. For the panning the Falcon 3046' plates were coated overnight with 0.25 mg/ml or 2 mg/ml tetanus toxoid dissolved in PBS or hydrogencarbonate pH 9.6.The wells were subsequently washed and residual protein binding sites were blocked with blocking solution at room temperature for 2 hours. The adsorption of the phagemid virions on the immobilized antigen and the washing and elution conditions were according to Marks et al (1991) or were taken from the protocol described by the ⟨Recombinant Phage Antibody System⟩of Pharmacia 4 consecutive rounds of panning were performed. After the fourth round of panning the eluted phagemid virions were added to exponentially growing TGI cells (Hoogenboom et al. 1991) and plated onampicilline containing LB plates. After overnight growth several colonies were grown individually in LB medium to midlogarithmic growing phase, and infected with M13K07 helper phage. The virions were prepared and tested for their binding activity against tetanus toxoid immobilised on microtiter plates. The presence of the virion binding to the immobilized antigen was revealed by ELISA using a Horse RadishPeroxidase/anti-M13 conjugate (Pharmacia). The percentage of binders was increasing after each round of panning. In the original library we found 3 clones out of 96 which showed binding with the immobilizes tetanus toxoid. This number was increased to 11, 48 and 80 after the first, second and third round of panning. All of the individual clones which were tested after the fourth round of panning were capable to recognize the antigen, as measured by ELISA (FIGURE 3). Ten positive clones were grown and tested by PCR to check the presence of an insert with the proper size of theV_{HH} gene, and their DNA was finally sequenced. The sequencing data revealed that two different clones were present among this set of 10 clones. The phasmid DNA of these clones was named pHEN4-αTT1 and pHEN4-αTT2, (The pHEN4-αTT2 phasmid DNA was deposited at the "BelgianCoordinated Collections of Microorganisms" BCCM/LMBP on January 31, 1995 under accession number LMBP3247), and it was shown that these two different clones contained a cDNA coding for a camel V_{HH} (FIGURE 4). Comparison of the amino acids in these clones with the camel V_{HH} clones analysed before (Muyldermans et al., 1994) clearly indicated that the anti-tetanus camel V_{HH} originated from a heavy chain immunoglobulin lack the CH1 domain and light chains. Especially the identity of the key residues at position 11 (Ser), 37 (Phe) and 45 (Arg or Cys) and 47 (Leu or Gly) prooved this statement (Muyldermans et al., 1994).

Production of soluble camel V_{HH} with anti-tetanus toxoid activity

The phasmid DNA of the two clones which scored positive in the tetanus toxoid ELISA were transformed into WK6 cells. These cells are unable to suppress the stopcodon present in the vector between the decapeptidetag and the gene III protein. The WK6 *E.coli* cells harboring the pHEN4-aTT1 or pHEN4-aTT2 phasmid were grown at 37°C in 1 liter of TB medium with 100 mgr ampicillin/ml and 0.1% glucose. When the cells reached an OD_{550} of 1.0 we harvested the cells by centrifugation at 5000 rpm, 10 minutes. The cell pellet was washed once in TB medium with ampicillin, but ommitting the glucoes. The cells were finally resuspended in 1 liter of TB medium with ampicillin (100 mgr/ml). We induced the expression of the camel V_{HH} domain by the addition of 1 mM IPTG and further growth of the cells at 28°C for 16 hours. The expressed proteins were extracted from the periplasmic space following the protocol described by Skerra and Plucthun (1988). We pelleted the *E.coli* cells by centrifugation at 4000g for 10 min. (4°C). The cells were resuspended in 10 ml TES buffer (0.2 M Tris-HCl pH 8.0, 0.5 mMEDTA, 0.5 M sucrose). The suspension was kept on ice for 2 hours. The periplasmic proteins were removed by osmotic shock by addition of 20 ml TES diluted 1/4 with water. The suspension was kept on ice for 1 hour and subsequently centrifuged at 12,000 g for 30 minutes at 4°C. The supernatant contained the expressed camel V_{HH} domain. The extract corresponding to 400 ml cell culture was applied under reducing conditions on a SDS/polyacrylamide protein gel. The extracted proteins were visualized in the SDS/polyacrylamide gels by Coomassie blue staining (FIGURE 5). A protein band with an apparent molecular weight of 16,000 D was clearly present in the E.coli cultures containing the recombinant clones and induced with IPTG. Alternatively, the presence of the camel V_{HH} proteins in the extract was revealed with IPTG. Alternatively,the presence of the camel V_{HH} proteins in the extract was revealed by Western blot using a specific rabbit anti-camel V_{HH} or rabbit anti-dromedary IgG serum or the anti-tag antibody.

We estimate from the band intensity observed in the Coomassie stained gel that more than 10 mg of the camel V_{HH} protein (non-purified) can be extracted from the periplasm of 1 liter induced *E.coli* cells.

For the purification of the anti-tetanus toxoid camel V_{HH} we concentrated the periplasmic extract 10 times by ultrafiltration (*Milipore* membrane with a cut off of 5000 Da). After filtration the concentrated extract from the pHEN4-aTT2 was separated according its molecular weight by gelfiltration on Superdex-75 (*Pharmacia*) (FIGURE 6) equilibrated with PBS (10 mM phosphate buffer pH7.2, 150 mM NaCl). The peak containing the anti-tetanus toxoid activity eluted at the expected molecular weight of 16,000 Da indicating that the protein behaved as a monomer and doesn't dimerize in solution. The fractions containing the pure V_{HH} (as determined by SDS-PAGE) were pooled and the concentration was measured spectrophotometrically using a calculated E_{280} (0.1%) of 1.2 and 2.3 respectively for the aTT1 and aTT2.

From the UV absorption at 280 nm of the pooled fraction we could calculate a yield of 6 mgr of purified protein per liter of bacterial culture. The purified protein could be further concentrated by ultrafiltration to 6 mgr/ml in PBS or water without any sign of aggregation, as seen on the UV spectrum.

Concerning the expression yield in *E.coli* it should be realized that at this stage we didn't try to optimize the expression or the protein extraction conditions. However, as the yield of the purified aTT2 camel V_{HH} reached 6 mgr per liter of bacterial culture, and as we obtained the soluble protein at a concentration of 6 mgr/ml, it is clear that the expression is comparable or better than other scFv's or F_{AB}'s expressed in *E.coli*. Furthermore, the solubility of the camel V_{HH} aTT2 is certainly better than that obtained for the mouse VH fragments. The yield and solubility is certainly in the range needed for most applications.

To proove the proper folding of the purified protein, the aTT2 was brought at a concentration of 3.9 x 10^{-6} M and used it for CD measurement (FIGURE 7). The CD spectrum is characteristic for a polypeptide with a b-pleated sheet folding as expected for a well structurated immunoglobulin fold (Johnson, 1990).

<u>The camel anti-tetanus toxoid V_{HH} affinity measurements</u>

The binding of the camel V_{HH} antibody to the tetanus toxoid immobilised on the microtiter plates was revealed by the successive incubation with firstly, the rabbit anti-camel V_{HH} or rabbit anti-dromedary IgG and secondly a goat anti-rabbit/alkaline phosphatase conjugated antibodies (Sigma). The apparent affinity of the camel V_{HH} proteins against tetanus toxoid was estimated by inhibition ELISA exactly as described by Persson et al. (1991) for the human anti-tetanus toxoid F_{AB} fragments they produced in <u>E.coli</u>

The specificity of the soluble camel V_{HH} for the tetanus toxoid was suggested from the ELISA experiments in which we competed the binding with free antigen was competed. An apparent inhibition constant of around 10^{-7}, 10^{-8} M was observed for both V_{HH} fragments (FIGURE 8). This compares favorable with the inhibition constants for the human anti-tetanus toxoid F_{AB} fragments cloned by Persson et al. (1991) which were in the range of 10^{-7} to 10^{-9} M.

The measurement of the affinity constant by ELISA is however, more reliable if determined according to the procedure of Friguet *et al.* (1987). With this protocol we found an affinity constant of 6.10^{7} M^{-1} and 2.10^{7} M^{-1} for the aTT1 and aTT2 respectively. These affinities are consistent with a specific V_{HH}-antigen interaction (the polyspecific antibodies generally bind their antigen with affinities of 10^{6} M^{-1} or less (Casali *et al.* 1989)).

Epitope recognition of αTT1 and αTT2.

Tetanus toxin consists of three domains. The C fragment binds to the neuronal cells, it is said to be the neurospecific binding domain. The B domain appears to be involved in the neuronal penetration of the A domain or L chain (Montecucco & Schiavo, 1993). The L chain is responsible for the intracellular activity.

The C fragment is the most immunogenic part of the tetanus neurotoxin, and a recombinant C fragment is commercially available (*Boehringer* and *Calbiochem*). We showed by ELISA that the aTT1 bacterial extract binds equally well both to the complete tetanus toxoid and to the recombinant C fragment. Therefore the epitope of this camel V_{HH} is present on the C fragment. By contrast, the αTT2 extract binds to the complete tetanus toxoid, but not to the C fragment. Therefore the αTT2 recognizes an epitope located on the A or B domain.

The *In vivo* neutralization of tetanus toxin toxicity.

The neutralizing activity of the purified camel αTT1 or αTT2 V_{HH} domains against tetanus toxin was tested. As a control, eight NMRI mice of 8 to 12 weeks (80 to 100 gr) were injected I.P. with 400 ngr tetanus toxin (SmithKline Beecham Biologicals) (= 10 times the LD50) in 0.1 ml PBS. To test the neutralizing activity of the camel V_{HH} αTT1 or αTT2 we preincubated 4 or 40 mgr of this purified recombinant protein with 400 ngr of the tetanus toxin in 0.1 ml of PBS for 30 minutes before I.P. injection into the mice. The survival of the mice was followed over a period of 2 weeks (FIGURE 9). It is clear that all mice injected with the tetanus toxin alone or in the presence of a non-specific purified camel V_{HH} (cVH21 of Muyldermans *et al.*, 1994) were killed within 3 days. The survival of the mice injected with the tetanus toxin was increased significantly by the co-injection of only 4 mgr of the purified camel αTT1 or αTT2. The survival was even more pronounced for the co-injection of tetanus toxin with 40 mgr of camel V_{HH}. It appears that the αTT1 had a slightly higher neutralizing activity than the αTT2. This could originate from its intrinsic higher affinity for binding the tetanus toxin (Simpson *et al.*, 1990). Alternatively it might result from the binding of the αTT1 V_{HH} to the fragment C of the tetanus toxin which inhibits more the toxic effect than the binding of the αTT2 to its epitope outside the C fragment.

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Montecucco & Schiavo (1993) TIBS 18, 324-329.
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Persson *et al.*, (1991) Proc.Natl.Acad.Sci USA 88, 2432-2436.
Sambrook *et al.*, (1989) Molecular Cloning CSHL Press
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The heavy-chain antibodies, such as those derived from camel, and their fragments present clear advantages over other antibodies or fragments thereof derived from other animals. These are linked to the distinctive features of the heavy chain antibodies and in particular the novel fragments which can be produced by proteolytic cleavage within the hinge of these heavy-chain antibodies to generate the V_{HH} and the $(V_{HH}h)2$ fragments. The VH domain of a heavy chain has distinct genetic entities which confer properties of solubility not found in VH fragments derived from conventional antibodies. This property, in addition to its small size and to the fact that the amino acid sequence of the framework region is very homologous to that of human, ensures a minimum of immunogenicity. These properties would allow repetitive treatment with heavy chain V_{HH} fragments for passive immunisation or antibody therapy. As mentioned above, V_{HH} and the $(V_{HH}h)2$ fragments can easily be produced by proteolytic cleavage of camel immunoglobulins or via recombinant DNA technology.

The most important field of passive immunisation is intoxication due to bacterial toxins and in particular acute intoxication or intoxication due to drug resistant bacteria. Passive immunisation or treatment by antibodies is justified in those cases where vaccination is unpractical or its effects short-lived. They are particularly justified for acute intoxication which if left untreated would have lethal or cripling effects.
The following list of indications is non-exhaustive:

- Tetanos due to infection by Clostridium tetani is an important post-trauma infection and current immunisations are not long lasting. It is also important in the veterinary field.
- Botulism due to ingestion of toxins produced by Clostridium Botulinum and related species.
- Gangrene due to infection by Clostridium.
- Necrotic Enteritis and Enterotoxemia in humans and livestock due to Clostridium Perfringens ingestion.
- Food poisoning due to Staphylococcal endotoxins in those cases where antibiotics are not recommended.
- Pseudomonas infection refractory to antibiotic treatment and in particular ocular infections where rapid intervention is warranted.
- Diphteria toxin infection
- Pasteurella and Yersinia infection causing lethal outcomes in human and livestock.
- Anthrax toxin produced by Bacillus Anthraxis and responsible for one of the five major livestock diseases.
- Infections due to other bacterial agents such as Neisseria or viral agents.

Furthermore, the relative resistance of the V_{HH} fragment to proteolytic cleavage by digestive enzymes (e.g. pepsin, trypsin) offer the possibility of treatment against important gut pathogens, such as Vibrio cholera and other vibrios, enterotoxic E.Coli, Salmonella species and Shigella or pathogens ingested with food such as Listeria.
Another major target for immunotherapy is in the treatment of intoxication due to bites or contact with toxic invertebrates and vertebrates. Among the invertebrates are sea anemonies, coral and jellyfish, spiders, beas and wasps, scorpions. In the vertebrates, the venemous snakes are of particular importance and in particular those belonging to the families of Viperidae, Crotalidae and lapidea.
Passive immunisation with partially purified immunoglobulins from immunized animals are allready being used. In developing countries, antitetanos and antidiphteria antisera are still produced on a very large scale, usually in horses. Anti-venom antibodies are produced, although on a much smaller scale, against venoms, especially snake venoms. Another field of application is in combination with the therapeutic use of toxins in medical or surgical practice where neurotoxins such as botulinum toxin are increasingly used.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(C) CITY: SINT-GENESIUS-RODE
(E) COUNTRY: BELGIUM
(F) POSTAL CODE (ZIP): 1640

(ii) TITLE OF INVENTION: VARIABLE FRAGMENTS OF IMMUNOGLOBULINS - USE FOR THERAPEUTIC OR VETERINARY PURPOSES

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: EP 95400932.0

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 357 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..357

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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TCT CTG AGA CTC TCC TGT GCG GCC TCT GGG GGA CAG ACC TTC GAT AGT        96
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             20                  25                  30

TAT GCC ATG GCC TGG TTC CGC CAG GCT CCA GGG AAG GAG TGC GAA TTG       144
Tyr Ala Met Ala Trp Phe Arg Gln Ala Pro Gly Lys Glu Cys Glu Leu
         35                  40                  45

GTC TCG AGT ATT ATT GGT GAT GAT AAC AGA AAC TAT GCC GAC TCC GTG       192
Val Ser Ser Ile Ile Gly Asp Asp Asn Arg Asn Tyr Ala Asp Ser Val
     50                  55                  60

AAA GGC CGA TTC ACC ATC TCC CGA GAC AAC GCC AAG AAC ACG GTA TAT       240
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr
 65                  70                  75                  80
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CTG CAA ATG GAC CGT CTG AAT CCT GAG GAC ACG GCC GTG TAT TAC TGT      288
Leu Gln Met Asp Arg Leu Asn Pro Glu Asp Thr Ala Val Tyr Tyr Cys
                 85                  90                  95

GCG CAA TTG GGT AGT GCC CGG TCG GCT ATG TAC TGT GCG GGC CAG GGG      336
Ala Gln Leu Gly Ser Ala Arg Ser Ala Met Tyr Cys Ala Gly Gln Gly
            100                 105                 110

ACC CAG GTC ACC GTC TCC TCA                                          357
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        115
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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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Tyr Ala Met Ala Trp Phe Arg Gln Ala Pro Gly Lys Glu Cys Glu Leu
         35                  40                  45

Val Ser Ser Ile Ile Gly Asp Asp Asn Arg Asn Tyr Ala Asp Ser Val
     50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr
 65                  70                  75                  80

Leu Gln Met Asp Arg Leu Asn Pro Glu Asp Thr Ala Val Tyr Tyr Cys
                 85                  90                  95

Ala Gln Leu Gly Ser Ala Arg Ser Ala Met Tyr Cys Ala Gly Gln Gly
            100                 105                 110

Thr Gln Val Thr Val Ser Ser
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(2) INFORMATION FOR SEQ ID NO: 8:

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(A) LENGTH: 381 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..381

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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ACC GTG GGC TGG TTC CGC CAG GTT CCA GGA AAG GAG CGC GAG GGG GTC     144
Thr Val Gly Trp Phe Arg Gln Val Pro Gly Lys Glu Arg Glu Gly Val
            155                 160                 165

GCG GGT ATC AGT AGT GGT GGC AGT ACC ACA GCC TAT TCC GAC TCC GTG     192
Ala Gly Ile Ser Ser Gly Gly Ser Thr Thr Ala Tyr Ser Asp Ser Val
        170                 175                 180

AAG GGC CGA TAC ACC GTC TCC CTT GAG AAC GCC AAG AAC ACT GTG TAT     240
Lys Gly Arg Tyr Thr Val Ser Leu Glu Asn Ala Lys Asn Thr Val Tyr
    185                 190                 195

CTA CTG ATA GAC AAC CTA CAA CCT GAA GAC ACT GCC ATA TAC TAC TGC     288
Leu Leu Ile Asp Asn Leu Gln Pro Glu Asp Thr Ala Ile Tyr Tyr Cys
200                 205                 210                 215

GCA GGA GTG AGC GGT TGG CGA GGG CGG CAG TGG CTG CTA CTG GCA GAG     336
Ala Gly Val Ser Gly Trp Arg Gly Arg Gln Trp Leu Leu Leu Ala Glu
                220                 225                 230

ACC TAT CGG TTC TGG GGC CAG GGG ACT CAG GTC ACC GTC TCC TCA         381
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(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

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Thr Val Gly Trp Phe Arg Gln Val Pro Gly Lys Glu Arg Glu Gly Val
         35                  40                  45

Ala Gly Ile Ser Ser Gly Gly Ser Thr Thr Ala Tyr Ser Asp Ser Val
     50                  55                  60

Lys Gly Arg Tyr Thr Val Ser Leu Glu Asn Ala Lys Asn Thr Val Tyr
 65                  70                  75                      80

Leu Leu Ile Asp Asn Leu Gln Pro Glu Asp Thr Ala Ile Tyr Tyr Cys
                 85                  90                  95

Ala Gly Val Ser Gly Trp Arg Gly Arg Gln Trp Leu Leu Leu Ala Glu
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Thr Tyr Arg Phe Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
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(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 98 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
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(B) LOCATION:27..31
(D) OTHER INFORMATION:/product= "OTHER"
/label= CDR1
/note= "This domain can contain up to 25 variable amino acid residues."

(ix) FEATURE:
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(B) LOCATION:33
(D) OTHER INFORMATION:/note= "Xaa at position 33 represents either Phe or Tyr"

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(B) LOCATION:41
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(A) NAME/KEY: Domain
(B) LOCATION:83..87
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/label= CDR3
/note= "This domain can contain up to 25 variable amino acid residues."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Xaa Xaa Xaa Xaa Xaa Trp
            20                  25                  30

Xaa Ala Gln Ala Pro Gly Lys Glu Xaa Glu Xaa Val Ser Xaa Xaa Xaa
        35                  40                  45

Xaa Xaa Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr
    50                  55                  60

Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys
65                  70                  75                  80

Ala Ala Xaa Xaa Xaa Xaa Xaa Trp Gly Gln Gly Thr Gln Val Thr Val
                85                  90                  95
```

Ser Ser

Claims

1. Variable fragment (V_{HH}) of a heavy chain of an immunoglobulin devoid of light chains, which is encoded by a nucleotide sequence obtainable by the following process:

   - treating blood lymphocytes or other appropriate cells of an animal of the Camelid family previously immunized with a determined antigen, in order to give access to their mRNA,
   - synthesizing a first strand of cDNA starting from the obtained mRNA,
   - contacting the obtained cDNA with at least two different primer oligonucleotides in conditions allowing their hybridization to at least two complementary nucleotide sequences contained in the cDNA, said primers comprising a BACK primer (back p1) having the following nucleotide sequence 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3' and a FOR primer (forp 1) replying to the following nucleotide sequence 5'-CGCCATCAAGGTACCGTTGA-3'
   - amplifying the DNA fragment located between the nucleotide sequence hybridized with said primers and,
   - recovering amplified DNA corresponding to bands of different size orders including:

     . a band of around 750 basepairs which is the amplified product of the variable heavy chain (V_H), CH1, hinge and part of CH2 region of a four-chain immunoglobin,
     . a band of around 620 basepairs which is the amplified product of the variable heavy-chain (V_{HH}), long hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG2,
     . a band of around 550 basepairs which is the amplified product of the variable heavy-chain (V_{HH}), short hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG3,

   - purifying the two shortest bands from agarose gel, for example by Gene Clean,
   - recovering the amplified DNA fragments containing nucleotide sequences encoding the V_{HH} fragments,
   - digesting the amplified products with restriction enzymes having target sites within the amplified fragments and/or in the nucleotide primers, for example with PstI and BstEII,
   - recovering the digested amplified DNA fragments,
   - ligating the amplified DNA fragments to a phasmid vector, for example in a pHEN4 vector, in conditions allowing the expression of the amplified fragments when the obtained recombinant vector is used to transform a host cell,
   - transforming a determined bacterial host cell for example an E. Coli cell with the obtained recombinant phasmid vector, and growing the cells on selective medium, to form a library,
   - infecting the obtained library of recombinant host cells after culture in an appropriate selective medium, with bacteriophages, for instance M13K07 bacteriophages to obtain recombinant phagemid virions,
   - incubating the recombinant host cells in conditions allowing secretion of recombinant phagemid virions particles containing the recombinant phasmid, for instance the pHEN4 phasmid packaged within the M13 virion.
   - isolating and concentrating the recombinant phagemid virions,
   - submitting the phagemid virions to several rounds of panning with the antigen of interest previously immobilized, in conditions allowing the adsorption of the phagemid virions on the immobilized antigen,
   - eluting the adsorbed phagemid virions, and growing them on appropriate cells,
   - amplifying the phagemid virions by infecting the cells with helper bacteriophage,
   - recovering the virions and testing them for their binding activity against the antigen of interest, for example by ELISA,
   - recovering the phagemid virions having the appropriate binding activity,
   - isolating the nucleotide sequence contained in the phasmid vector and capable of being expressed on the phagemid virions as a V_{HH} aminoacid sequence having the appropriate binding activity.

2. Variable fragment (V_{HH}) of a heavy chain of an immunoglobulin devoid of light chains, which is encoded by a nucleotide sequence obtainable by a process according to the one disclosed in claim 1, wherein an additional amplification step of the cDNA obtained from the mRNA is performed with oligonucleotide primers having respectively the following nucleotide sequences:

BACK primer: 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3'
FOR primer: 5'- CGCCATCAAGGTACCGTTGA-3'

3. Variable fragment (V_{HH}) of a heavy chain of an immunoglobulin devoid of light chains, which is encoded by a nucleotide sequence obtainable by a process according to the one disclosed in claim 1, wherein the amplification step of the cDNA obtained from the mRNA is performed with oligonucleotide primers having respectively the following nucleotide sequences:
BACK primer: 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3'
FOR primer 3: 5'- TGT CTT GGG TTC TGA GGA GAC GGT -3'
FOR primer 4: 5'-TTC ATT CGT TCC TGA GGA GAC GGT -3'

4. Variable fragment of a heavy chain of an immuglobulin devoid of light chains according to anyone of claims 1 or 2, encoded by a nucleotide sequence obtainable from blood lymphocytes or other appropriate cells of camelids wherein the camelids have been immunized with a determined antigen prior to the treatment of their blood lymphocytes or other appropriate cells.

5. Variable fragment of a heavy chain of an immuglobulin devoid of light chains according to anyone of claim 1 to 4 encoded by a nucleotide sequence obtainable from blood lymphocytes or other appropriate cells of camelids characterized in that the camelids have been previously immunized with an antigen which is a toxin of a bacteria or the corresponding toxoid.

6. Variable fragment of a heavy chain of an immuglobulin devoid of light chains according to claim 5 encoded by a nucleotide sequence obtainable from blood lymphocytes or other appropriate cells of camelids wherein the antigen is the tetanus toxoid of Clostridium tetani.

7. Variable fragment of a heavy chain of an immuglobulin devoid of light chains encoded by a nucleotide sequence according to claim 5 wherein the antigen is a bacterial toxin or toxoid chosen among those of the following bacteria: Clostridium, especially Clostridium Botulinum or Clostridium Perfringens, Staphylococcus, Pseudomonas, Pasteurella, Yersinia, Bacillus Anthracis, Neisseria, Vibrio, especially Vibrio cholera, enterotoxic E. Coli, Salmonella, Shigella, Listeria.

8. Variable fragment of a high chain of an immuglobulin devoid of light chains encoded by a nucleotide sequence obtainable from blood lymphocytes or other appropriate cells of camelids, wherein the camelids have been immunized with an antigen present in venom of animals.

9. Variable fragment of a high chain of an immuglobulin devoid of light chains according to claim 8, encoded by a nucleotide sequence wherein the antigen is a toxin or toxoid chosen among those produced by anemonies, coral, jellyfish, spiders, beas, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.

10. Variable fragment of a heavy chain of an immuglobulin devoid of light chains according to anyone of claims 1 to 3, characterized in that it comprises the following aminoacid sequence:

GluValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGlyGlySerLeuArgLeu

SerCysAlaAlaSerGly(**CDR1**)Trp(Phe Tyr)ArgGlnAlaProGlyLysGlu(Arg/Cys)

Glu(Gly/Leu)ValSer(**CDR2**)ArgPheThrIleSerArgAspAsnAlaLysAsnThrVal

TyrLeuGlnMetAsnSerLeuLysProGluAspThrAlaValTyrTyrCysAlaAla(**CDR3**)

TrpGlyGlnGlyThrGlnValThrValSerSer

wherein CDR1, CDR2 and CDR3 represent variable amino acid sequences providing for the recognition of a determined epitope of the antigen used for the immunization of Camelids, CDR1, CDR2 and CDR3 sequences comprising from 5 to 25 amino acid residues preferably CDR1 contains from 7 to 12 amino acid residues, CDR2 contains from 16 to 21 amino acid residues and CDR3 contains from 7 to 25 amino acid residues.

The camel V_{HH} specific amino acid residues Ser 11, Phe 37, Glu 44, Arg 45, Glu 46, Gly 47 are underlined.

11. Variable fragment of a heavy chain of an immuglobulin devoid of light chains coded by a nucleotide sequence present recombinant phasmid pHEN4-αTT2(WK6) deposited at the BCCM/LMBP under accession number LMBP3247.

12. Variable fragment of a heavy chain of an immunoglobulin devoid of light chains characterized in that it comprises the following αTT1 sequence:

GluValGlnLeuGlnAlaSerGlyGlyGly***Ser***ValGlnAlaGlyGlySerLeuArgLeu

SerCysAlaAlaSerGlyGlyGlnThrPhe***AspSerTyrAlaMetAla***Trp***Phe***ArgGln

AlaProGlyLysGluCysGluLeuValSer***SerIle***IleGly***AspAspAsnArgAsnTyr***

AlaAspSerValLysGlyArgPheThrIleSerArgAspAsnAlaLysAsnThrValTyr

LeuGlnMetAspArgLeuAsnProGluAspThrAlaValTyrTyrCysAlaGln***LeuGly***

SerAlaArgSerAlaMetTyrCysAlaGlyGlnGlyThrGlnValThrValSerSer

13. Variable fragment of a heavy chain of an immunoglobulin devoid of light chains characterized in that it comprises the following αTT2 aminoacid sequence.

GluValGlnLeuGlnAlaSerGlyGlyGly***Ser***ValGlnAlaGlyGlySerLeuArgLeu

SerCysThrAlaAlaAsnTyrAlaPheAspSerLysThrValGlyTrpPheArgGlnVal

ProGlyLysGlu***Arg***GluGlyValAla***GlyIleSerSerGlyGlySerThrThrAlaTyr***

SerAspSerValLysGlyArgTyrThrValSerLeuGluAsnAlaLysAsnThrValTyr

LeuLeuIleAspAsnLeuGlnProGluAspThrAlaIleTyrTyrCysAlaGly***ValSer***

GlyTrpArgGlyArgGlnTrpLeuLeuLeuAlaGluThrTyrArgPheTrpGlyGlnGly

ThrGlnValThrValSerSer

14. Variable fragment of a heavy chain of an immunoglobulin devoid of light chains according to anyone of claims 1 to 13 characterized in that it is linked to at least one further variable fragment of heavy chains of an immunoglobulin devoid of light chains according to anyone of claims 1 to 13, the V_{HH} fragments having the same antigen specificity.

15. Variable fragment of a heavy chains of an immunoglobulin devoid of light chains according to anyone of claims 1 to 13 characterized in that it is linked to at least one further variable fragment of heavy chains of an immunoglobulin devoid of light according to anyone of claims 1 to 13, the V_{HH} fragments having different antigen specificities.

16. Pharmaceutical composition, characterized in that it comprises an immunoglobulin variable fragment according to anyone of claims 1 to 13 in admixture with a physiologically acceptable vehicle.

17. Pharmaceutical composition according to claim 14 for the treatment by passive immunisation, of infection or acute intoxication by toxins such as those of Clostridium, especially Clostridium Botulinum or Clostridium Perfringens, Staphylococcus, Pseudomonas, Pasteurella, Yersinia, Bacillus Anthracis, Neisseria, Vibrio, especially Vibrio cholera, enterotoxic E. Coli, Salmonella, Shigella, Listeria or anemonies, coral, jellyfish, spiders, beas, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.

18. immunoglobulin variable fragment according to anyone of claims 2 to 10 for use for the treatment by passive immunisation, of infection or acute intoxication by toxins such as those of Clostridium, especially Clostridium Botulinum or Clostridium Perfringens, Staphylococcus, Pseudomonas, Pasteurella, Yersinia, Bacillus Anthracis, Neisseria, Vibrio, especially Vibrio cholera, enterotoxic E. Coli, Salmonella, Shigella, Listeria or anemonies, coral, jellyfish, spiders, beas, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.

19. Nucleotide sequence coding for a variable fragment (V_{HH}) of a heavy chain of an immunoglobulin devoid of light chains obtainable by the following process:

- treating blood lymphocytes or other appropriate cells of an animal of the Camelid family previously immunized with a determined antigen, in order to give access to their mRNA,

- synthesizing a first strand of cDNA starting from the obtained mRNA,
- contacting the obtained cDNA with at least two different primer oligonucleotides in conditions allowing their hybridization to at least two complementary nucleotide sequences contained in the cDNA, said primers comprising a BACK primer (back p1) having the following nucleotide sequence 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3' and a FOR primer (forp 1) replying to the following nucleotide sequence 5'-CGCCATCAAGGTACCGTTGA-3'
- amplifying the DNA fragment located between the nucleotide sequence hybridized with said primers and,
- recovering amplified DNA corresponding to bands of different size orders including:

  . a band of around 750 basepairs which is the amplified product of the variable heavy chain (V_H), CH1, hinge and part of CH2 region of a four-chain immunoglobin,
  . a band of around 620 basepairs which is the amplified product of the variable heavy-chain (V_{HH}), long hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG2,
  . a band of around 550 basepairs which is the amplified product of the variable heavy-chain (V_{HH}), short hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG3,

- purifying the two shortest bands from agarose gel, for example by Gene Clean,
- recovering the amplified DNA fragments containing nucleotide sequences encoding the V_{HH} fragments,
- digesting the amplified products with restriction enzymes having target sites within the amplified fragments and/or in the nucleotide primers, for example with PstI and BstEII,
- recovering the digested amplified DNA fragments,
- ligating the amplified DNA fragments to a phasmid vector, for example in a pHEN4 vector, in conditions allowing the expression of the amplified fragments when the obtained recombinant vector is used to transform a host cell,
- transforming a determined bacterial host cell for example an E. Coli cell with the obtained recombinant phasmid vector, and growing the cells on selective medium, to form a library,
- infecting the obtained library of recombinant host cells after culture in an appropriate selective medium, with bacteriophages, for instance M13K07 bacteriophages to obtain recombinant phagemid virions,
- harvesting the recombinant host cells, adsorbed with the bacteriophages,
- incubating the recombinant host cells in conditions allowing secretion of recombinant phagemid virions particles containing the recombinant phasmid, for instance the pHEN4 phasmid packaged within the M13 virion.
- isolating and concentrating the recombinant phagemid virions,
- submitting the phagemid virions to several rounds of panning with the antigen of interest previously immobilized, in conditions allowing the adsorption of the phagemid virions on the immobilized antigen,
- eluting the adsorbed phagemid virions, and growing them on appropriate cells,
- amplifying the phagemid virions by infecting the cells with helper bacteriophage,
- recovering the virions and testing them for their binding activity against the antigen of interest, for example by ELISA,
- recovering the phagemid virions having the appropriate binding activity,
- isolating the nucleotide sequence contained in the phasmid vector and capable of being expressed on the phagemid virions as a V_{HH} aminoacid sequence having the appropriate binding activity.

20. Nucleotide sequence coding for a variable fragment V_{HH} of a heavy chain of an immunoglobulin devoid of light chain, directed against an epitope of the tetanus toxin of Clostridium tetani, characterized in that it codes for an amino acid sequence according to claim 12 or 13.

21. Nucleotide sequence coding for a variable fragment V_{HH} of a heavy chain of an immunoglobulin devoid of light chain, directed against an epitope of the tetanus toxin of Clostridium tetani, characterized in that is comprises one of the following nucleotide sequences:

αTT1

```
         10        20        30        40        50        60
GAGGTGCAGCTGCAGGCGTCTGGGGAGGCTCGGTGCAGGCTGGAGGGTCTCTGAGACTC

         70        80        90       100       110       120
TCCTGTGCGGCCTCTGGGGGACAGACCTTCGATAGTTATGCCATGGCCTGGTTCCGCCAG

        130       140       150       160       170       180
GCTCCAGGGAAGGAGTGCGAATTGGTCTCGAGTATTATTGGTGATGATAACAGAAACTAT

        190       200       210       220       230       240
GCCGACTCCGTGAAAGGCCGATTCACCATCTCCCGAGACAACGCCAAGAACACGGTATAT

        250       260       270       280       290       300
CTGCAAATGGACCGTCTGAATCCTGAGGACACGGCCGTGTATTACTGTGCGCAATTGGGT

        310       320       330       340       350
AGTGCCCGGTCGGCTATGTACTGTGCGGGCCAGGGGACCCAGGTCACCGTCTCCTCA
```

αTT2

```
        10        20        30        40        50        60
GAGGTGCAGCTGCAGGCGTCTGGAGGAGGCTCGGTGCAGGCTGGAGGGTCTCTGAGGCTC

        70        80        90       100       110       120
TCTTGTACAGCCGCTAATTACGCCTTTGATTCCAAGACCGTGGGCTGGTTCCGCCAGGTT

       130       140       150       160       170       180
CCAGGAAAGGAGCGCGAGGGGGTCGCGGGTATCAGTAGTGGTGGCAGTACCACAGCCTAT

       190       200       210       220       230       240
TCCGACTCCGTGAAGGGCCGATACACCGTCTCCCTTGAGAACGCCAAGAACACTGTGTAT

       250       260       270       280       290       300
CTACTGATAGACAACCTACAACCTGAAGACACTGCCATATACTACTGCGCAGGAGTGAGC

       310       320       330       340       350       360
GGTTGGCGAGGGCGGCAGTGGCTGCTACTGGCAGAGACCTATCGGTTCTGGGGCCAGGGG

       370       380
ACTCAGGTCACCGTCTCCTCA
```

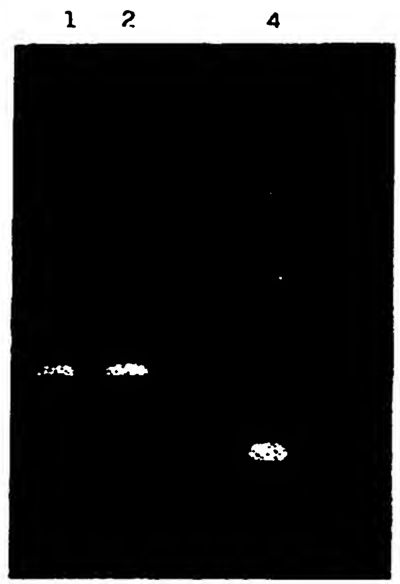
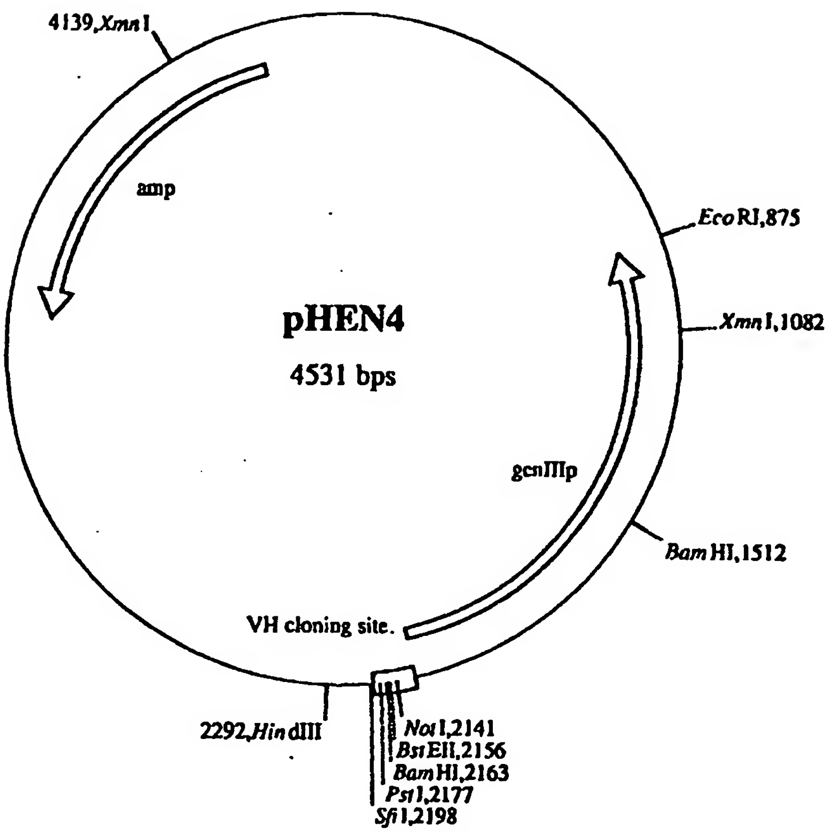


FIGURE 1



Pel B leader signal- - -I Q V Q L Q *(VH cloning site)* V T V S S
GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG CAG GAC CTC GAG GAT CCG GTC ACC GTC TCC AGC
Sfi I Pst I Bst EII

I- - - - - decapeptide tag - - - - - -I *I- - genIIIp*
GGC CGC TAC CCG TAC GAC GTT CCG GAC TAC GGT TCC GGC CGA GCA TAG ACT GTT
Not I Eag I amber

FIGURE 2

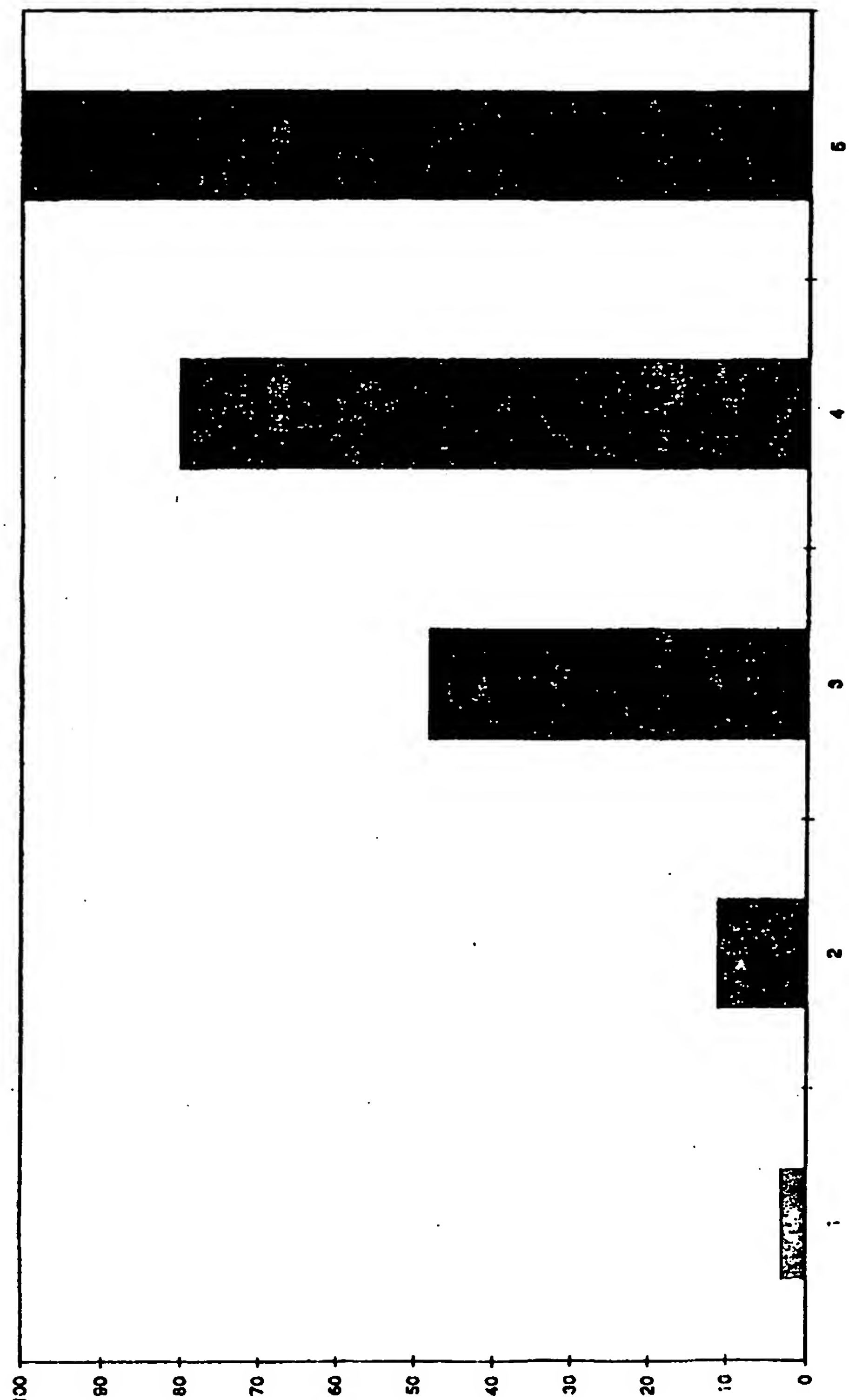


FIGURE 3

pHEN4-α TT1

```
        10        20        30        40        50        60
GAGGTGCAGCTGCAGGCGTCTGGGGGAGGCTCGGTGCAGGCTGGAGGGTCTCTGAGACTC
GluValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGlyGlySerLeuArgLeu

        70        80        90       100       110       120
TCCTGTGCGGCCTCTGGGGGACAGACCTTCGATAGTTATGCCATGGCCTGGTTCCGCCAG
SerCysAlaAlaSerGlyGlyGlnThrPheAspSerTyrAlaMETAlaTrpPheArgGln

       130       140       150       160       170       180
GCTCCAGGGAAGGAGTGCGAATTGGTCTCGAGTATTATTGGTGATGATAACAGAAACTAT
AlaProGlyLysGluCysGluLeuValSerSerIleIleGlyAspAspAsnArgAsnTyr

       190       200       210       220       230       240
GCCGACTCCGTGAAAGGCCGATTCACCATCTCCCGAGACAACGCCAAGAACACGGTATAT
AlaAspSerValLysGlyArgPheThrIleSerArgAspAsnAlaLysAsnThrValTyr

       250       260       270       280       290       300
CTGCAAATGGACCGTCTGAATCCTGAGGACACGGCCGTGTATTACTGTGCGCAATTGGGT
LeuGlnMETAspArgLeuAsnProGluAspThrAlaValTyrTyrCysAlaGlnLeuGly

       310       320       330       340       350
AGTGCCCGGTCGGCTATGTACTGTGCGGGCCAGGGGACCCAGGTCACCGTCTCCTCA
SerAlaArgSerAlaMETTyrCysAlaGlyGlnGlyThrGlnValThrValSerSer
```

FIGURE 4A

pHEN4-αTT2 --

10 20 30 40 50 60

GAGGTGCAGCTGCAGGCGTCTGGAGGAGGCTCGGTGCAGGCTGGAGGGTCTCTGAGGCTC

GluValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGlyGlySerLeuArgLeu

70 80 90 100 110 120

TCTTGTACAGCCGCTAATTACGCCTTTGATTCCAAGACCGTGGGCTGGTTCCGCCAGGTT

SerCysThrAlaAlaAsnTyrAlaPheAspSerLysThrValGlyTrpPheArgGlnVal

130 140 150 160 170 180

CCAGGAAAGGAGCGCGAGGGGGTCGCGGGTATCAGTAGTGGTGGCAGTACCACAGCCTAT

ProGlyLysGluArgGluGlyValAlaGlyIleSerSerGlyGlySerThrThrAlaTyr

190 200 210 220 230 240

TCCGACTCCGTGAAGGGCCGATACACCGTCTCCCTTGAGAACGCCAAGAACACTGTGTAT

SerAspSerValLysGlyArgTyrThrValSerLeuGluAsnAlaLysAsnThrValTyr

250 260 270 280 290 300

CTACTGATAGACAACCTACAACCTGAAGACACTGCCATATACTACTGCGCAGGAGTGAGC

LeuLeuIleAspAsnLeuGlnProGluAspThrAlaIleTyrTyrCysAlaGlyValSer

310 320 330 340 350 360

GGTTGGCGAGGGCGGCAGTGGCTGCTACTGGCAGAGACCTATCGGTTCTGGGGCCAGGGG

GlyTrpArgGlyArgGlnTrpLeuLeuLeuAlaGluThrTyrArgPheTrpGlyGlnGly

370 380

ACTCAGGTCACCGTCTCCTCA

ThrGlnValThrValSerSer

FIGURE 4B

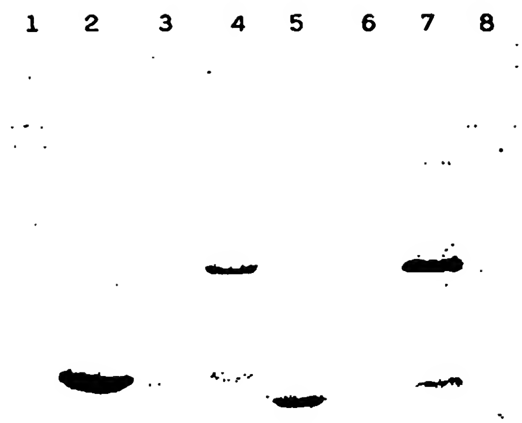


FIGURE 5

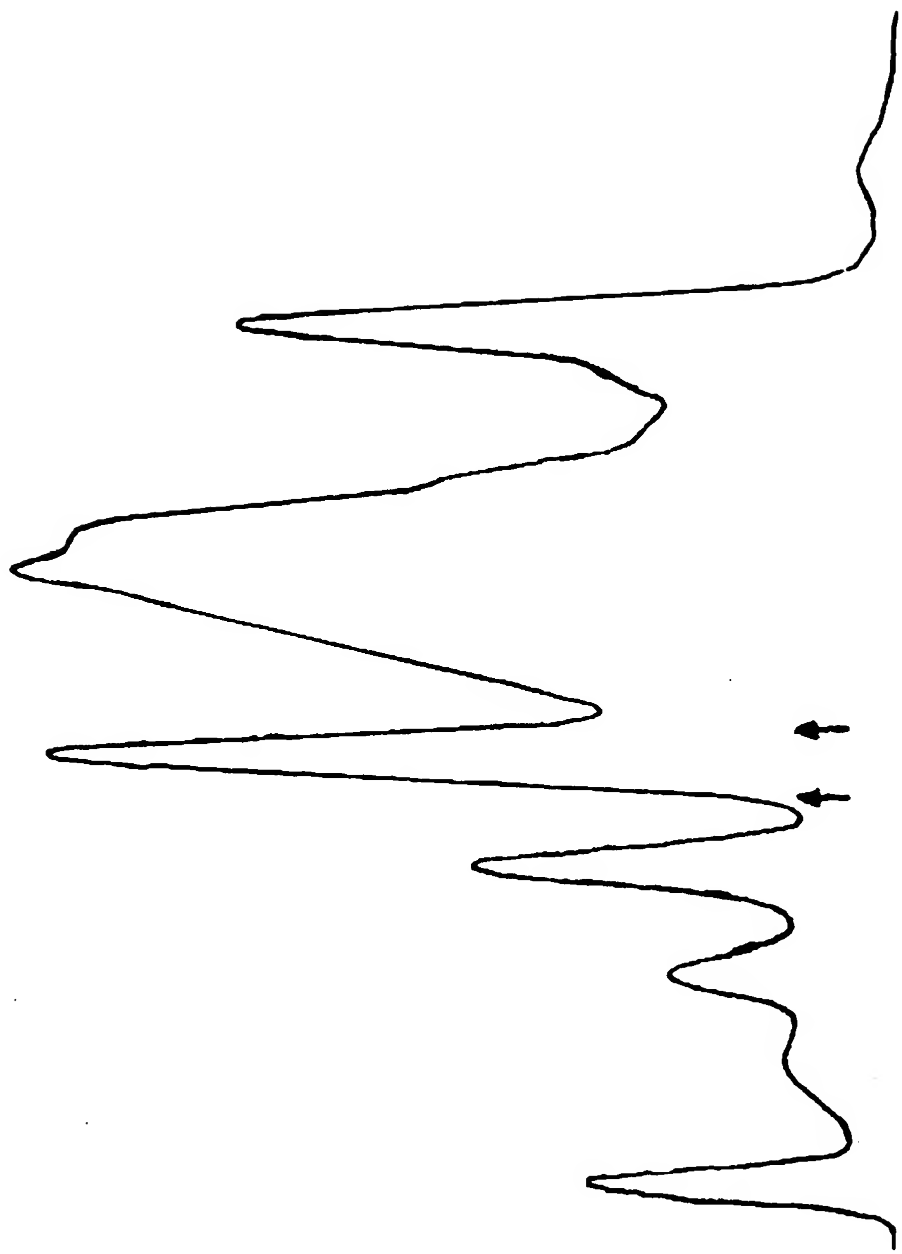
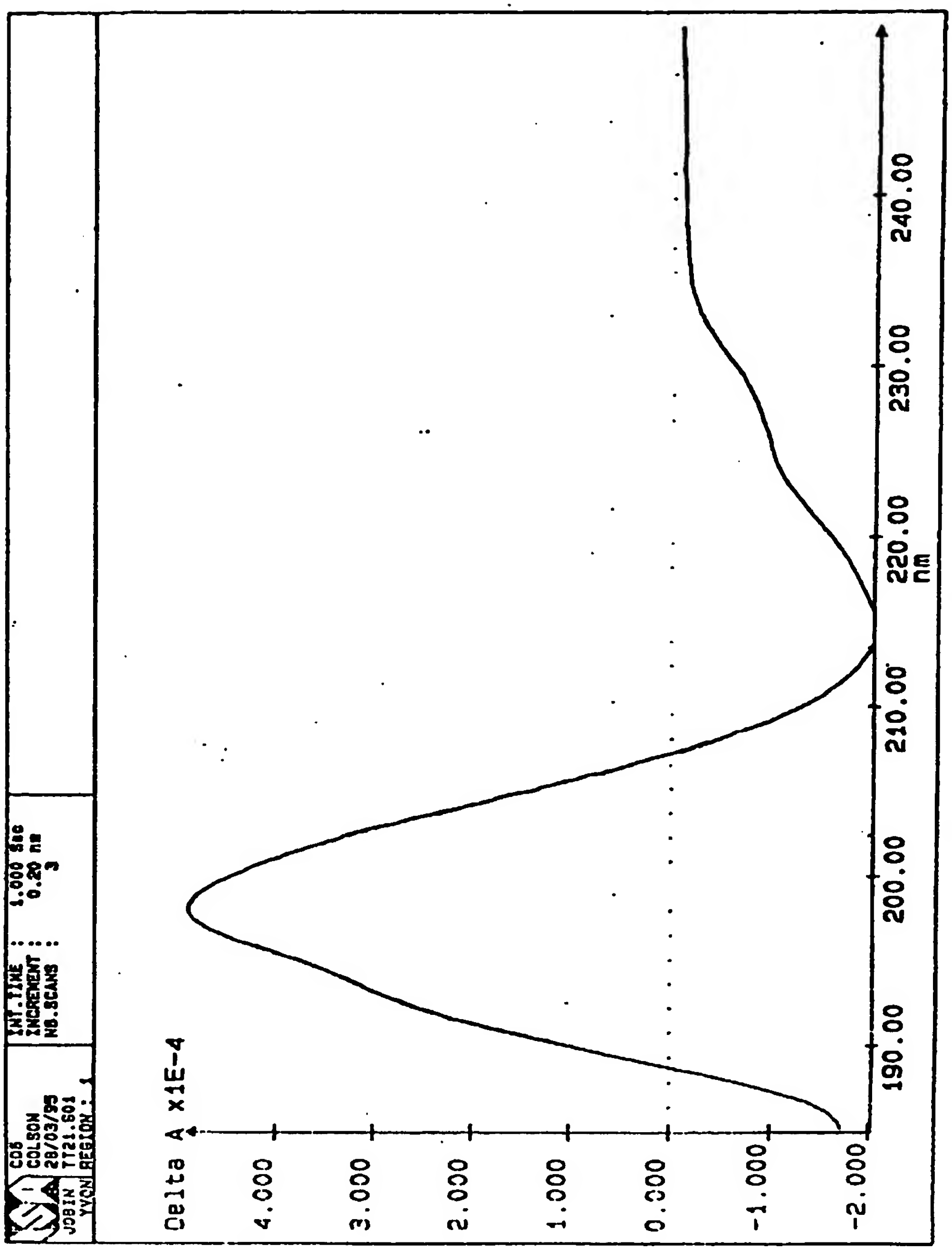


FIGURE 6



INT.TIME : 1.000 Sec
INCREMENT : 0.20 nm
NB.SCANS : 3
COLSON
28/03/95
TT21.601
REGION : 1
Delta A x1E-4
4.000
3.000
2.000
1.000
0.000
-1.000
-2.000
190.00
200.00
210.00
220.00
230.00
240.00
nm

FIGURE 7

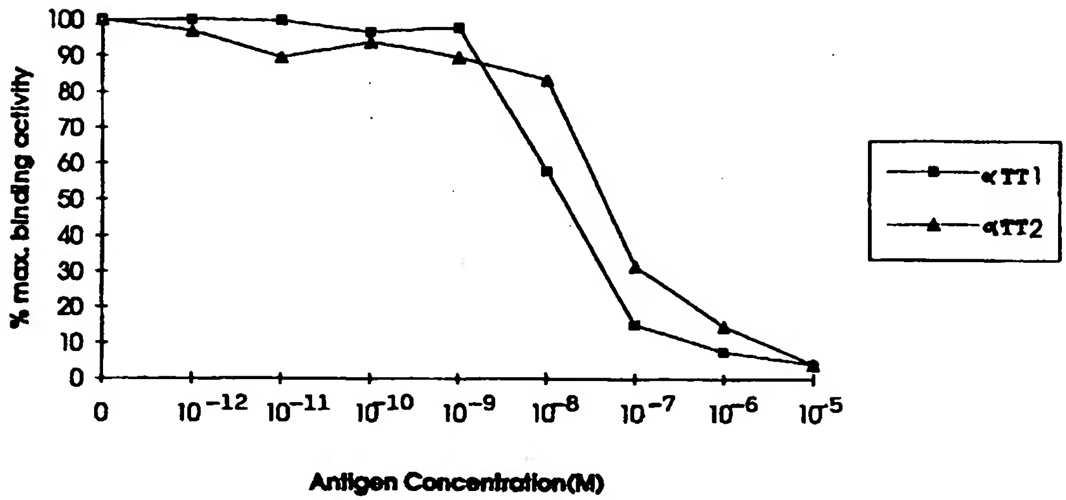

% max. binding activity
100
90
80
70
60
50
40
30
20
10
0
0
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Antigen Concentration(M)
αTT1
αTT2

FIGURE 8

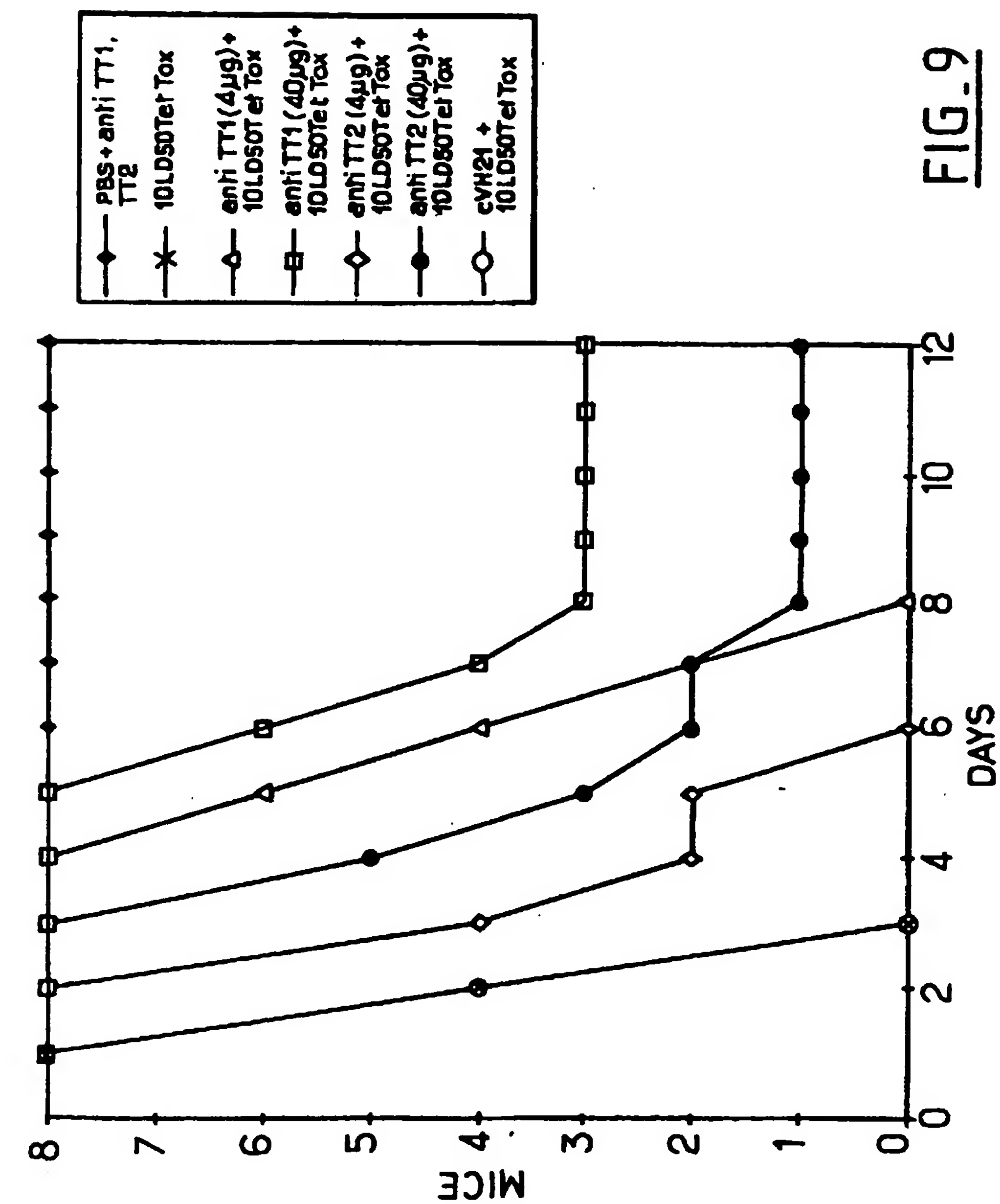

PBS + anti TT1, TT2
10LD50Tet Tox
anti TT1(4µg)+ 10LD50Tet Tox
anti TT1 (40µg)+ 10LD50Tet Tox
anti TT2 (4µg)+ 10LD50Tet Tox
anti TT2 (40µg)+ 10LD50Tet Tox
cVH21 + 10LD50Tet Tox
MICE
0
1
2
3
4
5
6
7
8
DAYS
0
2
4
6
8
10
12

FIG_9

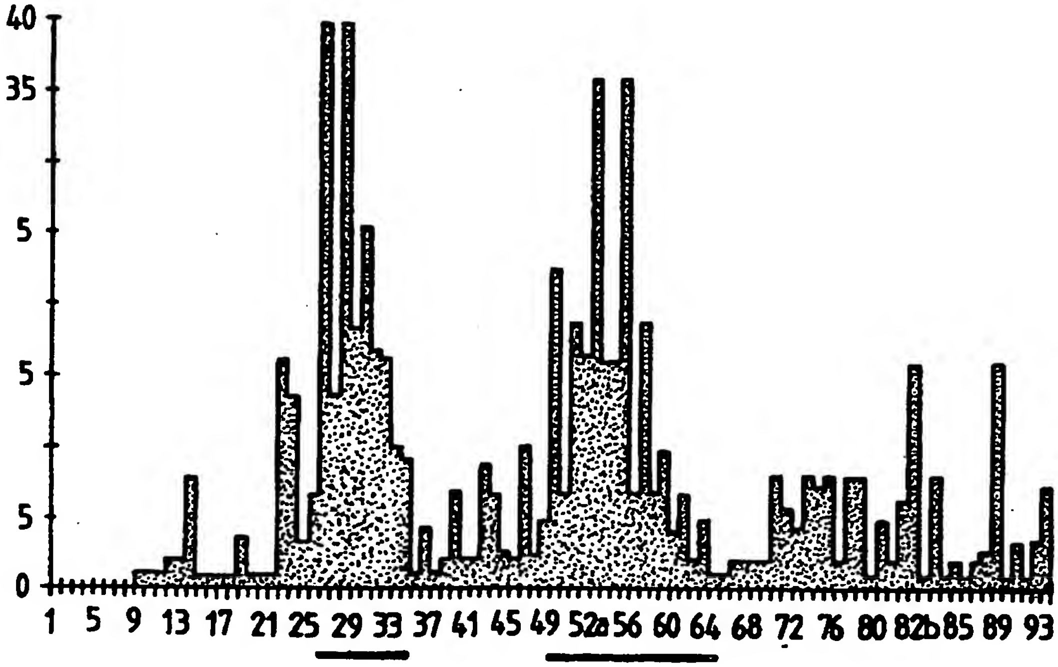

40
35
5
5
5
0
1 5 9 13 17 21 25 29 33 37 41 45 49 52a 56 60 64 68 72 76 80 82b 85 89 93

FIG_10

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EUROPEAN SEARCH REPORT

Application Number
EP 95 40 0932

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,A	WO-A-94 04678 (C. CASTERMAN ET AL.) 3 March 1994 * claims * ---	1-21	C12N15/13 C07K16/00 A61K39/395
D,A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 87, no. 20, October 1990 WASHINGTON, DC, USA, pages 8095-8099, R. MULLINAX ET AL. 'Identification of human antibody fragment clones specific for tetanus toxoid in a bacteriophage lambda immunoexpression library.' * the whole document * ---	1-6, 16-20	
D,A	PROTEIN ENGINEERING, vol. 7, no. 9, September 1994 OXFORD, GB, pages 1129-1135, S. MUYLDERMANS ET AL. 'Sequence and structure of Vh domain from naturally occurring camel heavy chain immunoglobulins lacking light chains.' * abstract * * figures * ---	10	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12N C07K A61K
A	FEBS LETTERS, vol. 339, no. 3, 21 February 1994 AMSTERDAM, NL, pages 285-290, J. DAVIES ET AL. ''Camelising' human antibody fragments: NMR studies on VH domains.' * the whole document * ---	10	
A	WO-A-93 01288 (DEUTSCHES KERNFORSCHUNGSZENTRUM STIFTUNG DES ÖFFENTLICHEN RECHTS) 21 January 1993 * the whole document * --- -/--	1,19	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	13 October 1995	Nooij, F

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 95 40 0932

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	WO-A-94 25591 (UNILEVER N.V.) 10 November 1994 * examples * * claims *	1-21	
A	AMERICAN JOURNAL OF VETERINARY RESEARCH, vol. 50, no. 8, August 1989 CHICAGO, IL, USA, pages 1279-1281, J. PAUL-MURPHY ET AL. 'Immune response of the llama (Lama glama) to tetanus toxoid vaccination.' * abstract *	4-6,20	
T	BIO/TECHNOLOGY, vol. 13, no. 5, May 1995 USA, pages 475-479, J. DAVIES ET AL. 'Antibody VH domains as small recognition units.' * the whole document *	1-21	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	13 October 1995	Nooij, F

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D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document